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Positional and geometrical anionic isomer separations by capillary electrophoresis-electrospray ionization-mass spectrometry

Capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) was applied to the analysis of polar positional and geometrical anionic isomers. Since the investigated positional and geometrical anionic isomers have different pK_a values, they could be separated by CE-ESI-MS under simple analytical conditions using a bare fused-silica capillary and volatile ammonium acetate buffer after optimizing buffer pH. *Ortho*-, *meta*-, *para*-hydroxybenzoate positional isomers were completely separated on a fused-silica capillary with 20 mM ammonium acetate buffer at pH 10.0, and *cis*-, *trans*-cyclohexane dicarboxylate geometrical isomers could be also separated with 20 mM ammonium acetate buffer at pH 4.0. Several analytical parameters affecting ESI-MS sensitivity were also investigated. It was found that both running buffer pH and sheath liquid pH had significant effects on the selectivity and the sensitivity on CE-ESI-MS analysis while sheath flow rate and other parameters had little influence. Under optimized conditions, linearity, detection limit, and repeatability of the analysis of hydroxybenzoate isomers were examined, and good results were obtained. It was found that the method presented in this paper is a simple, robust, and cost-effective method for simultaneous analysis of positional and geometrical anionic isomers as well as of other small anionic compounds.

Keywords: Anionic isomers / Capillary electrophoresis-mass spectrometry / Carboxylic acids / Electrospray ionization / Positional and geometrical isomers DOI 10.1002/elps.200410122

1 Introduction

For chromatographic separations of positional and geometrical isomers, some special techniques are usually necessary to separate them successfully since such isomers are similar in chemical and physical properties. For instance, fatty acidic isomers are relatively hydrophobic and they are easily retained on a common stationary phase. However, a molecular recognition property should be incorporated into the separation process to enhance the selectivity of their isomer separation. Therefore, some specially designed stationary phases, mobile phase additives, and derivatization techniques have been proposed for gas chromatography (GC) and liquid chromatography (LC) analysis. Most reports for isomer analysis by LC and GC dealt with relatively large hydrophobic molecules. On the other hand, so far there are few reports on direct analysis of highly polar small ionic isomers without derivatization procedure. For enantioresolution of underivatized amino acids, the use of a teicoplanin-bonded chiral stationary phase (CSP) as an alternative to crown ether and/or ligand-exchange CSPs was proposed by Armstrong *et al.* [1–4]. The teicoplanin CSP has been accepted due to the excellent resolution and the convenient eluent composition for UV and MS detection, however, the application was focused on mainly chiral separations of various types of amino acids, peptides, and aromatic carboxylic acids. Highly polar small acidic compounds without amine moiety have not been applied so far [1–6]. For GC analysis, laborious derivatization steps are necessary to enhance the volatility for such highly polar and nonvolatile compounds. On the other hand, for LC analysis, it is relatively difficult to retain highly polar compounds on common reversed-phase columns. To achieve successful separation of ionic compounds by LC, a number of separation parameters, such as the type of column, type of ion-pairing reagent and its concentration, mobile phase composition (especially buffer pH), and gradient conditions have to be optimized. However, these steps are very time-consuming. Although ion chromatography (IC) is a powerful method for separating such ionic compounds, the mobile phase used for IC is generally incompatible with ESI-MS detection, which pro-

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vides qualitative information. Therefore, a novel analytical method, which is suitable for the analysis of highly polar small compounds, is indispensable.

Capillary electrophoresis (CE) has been developed and regarded as one of the most powerful separation techniques for highly polar ionic compounds. CE separation is based on the ionic character and aquo-ion size of the solute, the separation principle being different from that of chromatography [7]. Therefore, CE would be suitable for ionic isomer analysis. Although CE will provide an extremely excellent separation efficiency and resolution, the obtainable sensitivity is generally inferior to LC due to the limited light path length of the UV detector, which is the most common detector in CE. Even if UV spectra are acquired by a diode array detector, they will not give enough information for compound identification in many cases. Fluorescence detection (FLD) and electrochemical detection (ECD) are superior in sensitivity and selectivity compared with UV detection. However, their application areas are limited and a derivatization step with fluorescent or electroactive reagent is required in some cases.

Recently, CE coupled with ESI-MS (CE-ESI-MS), which provides molecular weight information and/or chemical structural information, has been gaining attention because of its superior sensitivity and selectivity. So far, most reports for CE-MS focused on small cation analysis, such as peptides, pesticides, herbicides, drugs, catecholamines, and amino acids [8–14]. In the earlier CE-MS researches, only few researchers have reported anion analysis. For small anion analysis by CE-MS, a buffer modifier or a cationic polymer-coated capillary column has been used to reverse the direction of the electroosmotic flow (EOF) to conduct stable CE-MS analysis [15–18]. However, the use of a buffer modifier will deteriorate ES-ionization efficiency and decrease the ES-ionization sensitivity. Also, polymer-coated columns are generally less durable and more expensive compared with a bare fused-silica capillary, and they can be used only within a limited pH range. Therefore, we previously reported the development of a simple, robust, and cost-effective CE-MS method using a bare fused-silica capillary and simple composition of volatile buffer solution for the analysis of small carboxylic anions [19, 20]. Generally, small anions migrate to the opposite direction of the EOF. However, the anions are carried with a strong EOF to the MS detection side under the examined highly alkaline buffer conditions. In our previous study, the method was only applied to the analysis of a few carboxylic acids with a pH 10.0 running buffer; optimization of the running buffer pH was not investigated, however, it is expected that it would be able to apply the method to other small anionic isomers. Therefore, in this study, we investigated the applicability of the CE-MS method using a fused-silica capillary and alkaline volatile buffer solution to anionic isomer analysis. In particular, factors affecting the selectivity and the sensitivity for the analysis of anionic positional and geometrical isomers were investigated. Hydroxybenzoate positional isomers and cyclohexane dicarboxylate *cis-trans* geometrical isomers were chosen as representatives of anionic isomers in this study. The results for the analysis of small anionic isomers by CE-MS are presented.

2 Materials and methods

2.1 Chemicals and materials

Water used in the experiments was deionized and purified by a Milli-Q purification system (Millipore, Bedford, MA, USA). Analytical-grade methanol, ammonium acetate, ammonium formate, acetic acid, and 25% ammonium hydroxide solution were purchased from Wako (Osaka, Japan), and all other reagents were of analytical grade from Tokyo-Kasei (Tokyo, Japan). The stock standard solutions of the investigated compounds (*o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *cis*-cyclohexane-1,2-dicarboxylic acid, and *trans*-cyclohexane-1,2-dicarboxylic acid) were prepared by dissolving each in 1% ammonium hydroxide solution to give a concentration of 1 mg/mL. The test mixtures were prepared by diluting the stock solutions with Milli-Q water. The fused-silica capillary (50 μm ID, 375 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ, USA). A flat surface of the capillary was obtained by using the capillary column cutter with a diamond blade from Agilent Technologies (Waldbronn, Germany).

2.2 CE-ESI-MS instrumentation

The CE-ESI-MS system was from Agilent Technologies and used in the all experiments. An Agilent CE-ESI-MS sprayer kit (G1607A) and Agilent CE ESI-MS adapter kit (G1603A) were used to conjunct the Agilent CE system (G1600A) to the Agilent 1100 series single quadruple mass spectrometer (G1946D). An Agilent 1100 series online degasser and a binary pump equipped with a 1:100 splitter were employed to deliver the sheath flow. Agilent ChemStation software was used for the entire system control, data acquisition, and data analysis.

2.3 Experimental conditions

All experiments were carried out using a fused-silica capillary with 50 μm ID × 60 cm (total length). 20 mM ammonium acetate in water was used as the run electro-

lyte solution. The pH values of the electrolyte were adjusted to 3.5, 4.0, 4.5, 9.0, 9.5, and 10.0, respectively, with 1% acetic acid or 1% ammonium hydroxide. Prior to the first use, a new capillary was conditioned by flushing with the running buffer for 20 min. Between runs, the capillary was flushed with the fresh running buffer solution for 4 min. Hydrodynamic injection with 50 mbar pressure for 6.0 s was used for sample loading. The CE voltage was set at +20 kV and the capillary temperature was controlled to 25°C.

2.4 CE-ESI-MS conditions

As the sheath liquid solutions, three types were prepared: 0.5% formic acid in 50% v/v methanol/water (pH 3.0), 5 mM ammonium acetate in 50% v/v methanol/water (pH 7.0), and 5 mM ammonium hydroxide in 50% v/v methanol/water (pH 10.0). For MS detection, the ESI-negative ionization mode was selected. The MS capillary voltage was set to 3500 V and the MS fragmentor voltage was set to 100 V throughout this study. The nebulizer gas, the drying gas flow rate, and the temperature were set to 10 psi, 10 L/min, 300°C, respectively.

3 Results and discussion

3.1 Selection of running buffer

It is well-known that the selection of the running buffer has a significant impact on CE separations. Therefore, optimizing the pH of the running buffer is the most important parameter to obtain sufficient separation selectivity. In this study, ammonium acetate buffer was chosen for all experiments since it has sufficient volatility and does not suppress the ESI process. As a preliminary experiment, ammonium formate buffer was also investigated. However, the use of acetate buffer gave a better peak shape for the anionic isomers studied in this paper. In addition, the effects of the buffer concentration on the separation and detection sensitivity for CE-MS analyses of anionic isomers were investigated with ammonium acetate buffer in the range of 10–40 mM. As a result, the use of 20 mM ammonium acetate buffer gave reproducible migration times and reasonable MS sensitivity for each anionic isomer. At higher buffer concentration (*e.g.*, 30 or 40 mM), MS sensitivity was decreased and the analysis time was increased. Therefore, 20 mM ammonium acetate buffer was used as running buffer in the following experiments.

To evaluate the effects of buffer pH on CE selectivity and MS sensitivity, *o*-, *m*-, and *p*-hydroxybenzoate isomers were analyzed in the pH range of 3.5–10.0. Figure 1 shows SIM electropherograms obtained with the analysis of a standard solution of *o*-, *m*-, and *p*-hydroxybenzoic acid at 1 mg/L concentration each with acidic ammonium acetate buffer (pH 3.5–4.5). The SIM electropherograms were obtained by monitoring the *m/z* 137 ion, which is corresponding to its deprotonated ion form, $[M-H]^-$. The SIM electropherograms obtained with alkaline buffer solutions (pH 9.0–10.0) are shown in Fig. 2. The migration order of hydroxybenzoate isomers in the respective pH buffers could be explained with their dissociation constant (pK_a) values shown in Table 1. When acidic buffer solutions were used, the isomers were detected in the order of *p*-, *m*-, and *o*-form (Fig. 1). For instance, according to the pK_{a1} values listed in Table 1, the *o*-isomer has the strongest anionic character in acidic buffer solutions and has the higher electrophoretic mobility towards the anode (sample inlet end). Therefore, the *o*-isomer could be detected last.

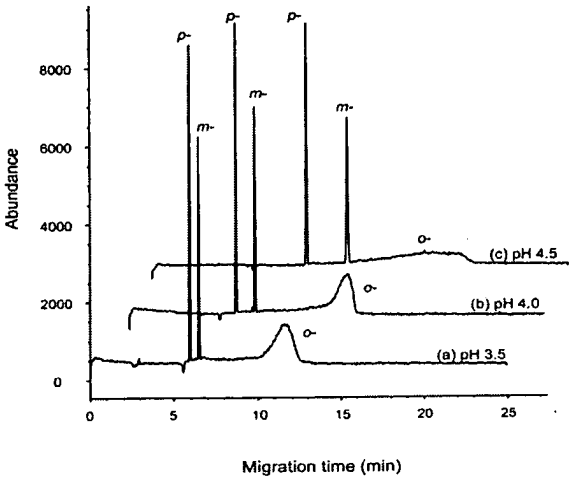


Figure 1. SIM electropherograms of *o*-, *m*-, *p*-positional isomers with acidic running buffer. Conditions: capillary, fused silica, 50 μm ID × 60 cm total length; running buffers, 20 mM ammonium acetate (a) pH 3.5, (b) pH 4.0, (c) pH 4.5; applied voltage, 20 kV; capillary temperature, 25°C; injection, 50 mbar pressure for 6.0 s; ionization, ESI-negative; sheath liquid, 5 mM ammonium hydroxide in methanol/water (50/50 v/v), flow rate of the sheath liquid, 6 μL/min; sample concentration, 1 mg/L each.

Table 1. pK_a values of hydroxybenzoic acid isomers[a)]

Substance		pK_1	pK_2
Hydroxybenzoic acid:	*ortho-*	3.00	12.38
	meta-	4.08	9.85
	para-	4.58	9.23

a) pK_a values were taken from *Lange's Handbook of Chemistry*, 11th ed., McGraw-Hill, New York 1973.

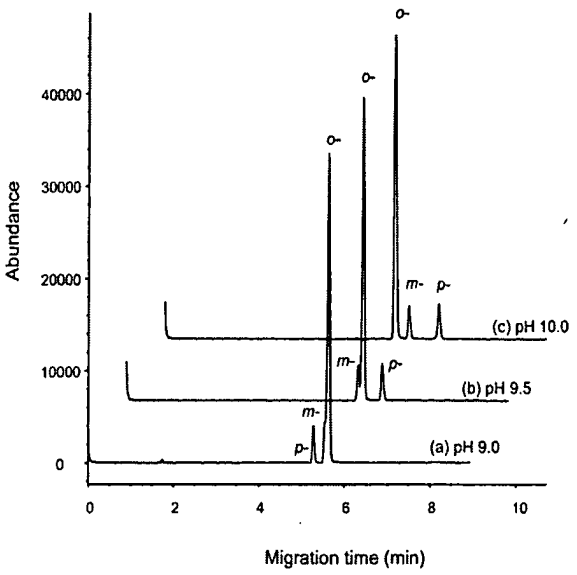


Figure 2. SIM electropherograms of *o*-, *m*-, *p*-positional isomers with alkaline running buffer. Condition: capillary, fused silica 50 μm ID × 60 cm total length; running buffers, 20 mM ammonium acetate (a) pH 9.0, (b) pH 9.5, (c) pH 10.0. Other conditions as in Fig. 1.

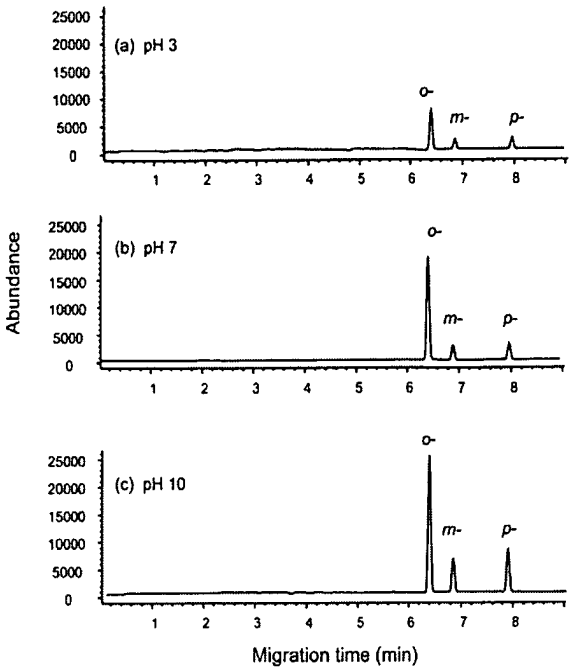


Figure 3. Effect of sheath liquid pH on negative ESI sensitivity of hydroxybenzoates. (a) 0.5% formic acid in methanol/water (50/50 v/v), pH 3; (b) 5 mM ammonium acetate in methanol/water (50/50 v/v), pH 7; (c) 5 mM ammonium hydroxide in methanol/water (50/50 v/v), pH 10; sample concentration, 1 mg/L each. Other analytical conditions as in Fig. 1.

All isomers were completely separated in acidic buffer between pH 3.5 to 4.5, however, only the *o*-isomer showed severe peak-broadening. The peak of the *o*-isomer could not be clearly observed at pH 4.5. In contrast, good symmetric peaks were obtained for all isomers in alkaline buffers at pH 9.0–10.0, as shown in Fig. 2. In particular, excellent resolution and symmetric peaks were obtained when pH 10.0 buffer was used as the running solution. Moreover, the detection sensitivity of the *o*-isomer was significantly improved (by approximately 30-fold) under alkaline conditions. The sensitivity enhancement of the *o*-isomer is probably due to the peak shape improvement and the facilitated dissociation of the anion. As can be seen in the figures, selection of the buffer solution is an important parameter to enhance the sensitivity of the analysis of anions in CE-MS.

3.2 Effect of sheath liquid on ESI-MS sensitivity

Since the sheath liquid flow is the dominant flow for CE-MS analysis, it is expected that the sheath liquid pH affects to ESI-MS sensitivity. Therefore, the effect of sheath liquid pH on ESI-MS sensitivity was tested by using three different pH solutions: 0.5% acetic acid in water/methanol (pH 3.0), 5 mM ammonium acetate in water/methanol (pH 7.0), and 5 mM ammonium hydroxide in water/methanol (pH 10.0). Figure 3 shows the effect of the sheath liquid pH on the negative ESI sensitivities of hydroxybenzoates. It was found that the use of 5 mM ammonium hydroxide in 50% v/v methanol/water (pH 10.0) as the sheath liquid had a significant effect on the sensitivity enhancement of these anionic compounds compared with the lower-pH sheath liquid solutions. The peak heights for all isomers in the pH 10.0 sheath solution were approximately 3.5-fold larger than that in pH 3.0 solution. Under alkaline condition, the carboxylic group of the benzoate would be effectively deprotonated, thereby faciliting the ionization of benzoate in the ESI process in negative mode. Also, phenolic hydroxyl might be contributing to sensitivity enhancement under alkaline condition. However, their doubly charged ions were not observed under the examined condition. Based on these results, it was found that the use of alkaline sheath liquid is a proper choice in CE-MS analysis of small anionic compounds for sensitivity enhancement.

Another important parameter, the effect of the sheath liquid flow rate on the ESI sensitivity was also investigated in the range of 4–10 μL/min. It was found that the sheath

liquid flow rate had only a little effect on the sensitivity under the examined conditions. At higher flow rates, the sensitivity was slightly decreased probably due to a dilution effect with the sheath liquid. At the lowest flow rate (4 μL/min), the sensitivity was also somewhat decreased though the reason is unclear. Highly stable and sensitive analysis could be achieved at 6–8 μL/min. Therefore, the following experiments were performed at 6 μL/min.

3.3 Method validation

The developed method was validated in terms of repeatability of migration time and peak area, detection limit, and linearity. The results are summarized in Table 2. For more precise peak identification, benzoate was used as the internal standard (IS), and the relative migration time of the isomers to IS was used for calculation of repeatability of migration time for the isomers. The repeatability was calculated from six consecutive runs (run-to-run) and three consecutive days (day-to-day) by analyzing 1 mg/L of each standard solution of hydroxybenzoates and benzoate. The obtained relative standard deviation (RSD) values of the relative migration time for the isomers were less than 0.1% (run-to-run) and 0.5% (day-to-day). For the peak area, acceptable results were also obtained. The limit of detection (LOD) (S/N = 3) was calculated from the SIM electropherogram of a 50 μg/L standard mixture. The LODs were 5 μg/L for the *o*-isomer, 20 μg/L for the *m*-isomer, and 20 μg/L for the *p*-isomer, respectively. For each hydroxybenzoate, excellent linearity was observed in the concentration range of 0.05–1.0 mg/L and the correlation coefficient was more than 0.999.

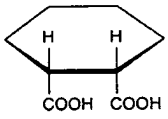
Table 2. Repeatability of relative migration time, peak area, correlation coefficient, and LOD for the analysis of hydroxybenzoate isomers

Parameter	*o*-	*m*-	*p*-
%RSD for relative migration time (run-to-run)[a]	0.02	0.09	0.07
%RSD for relative migration time (day-to-day)[b]	0.32	0.47	0.24
%RSD for peak area (run-to-run)[a]	1.3	2.0	1.6
%RSD for peak area (day-to-day)[b]	4.5	6.2	6.4
Correlation coefficient[c]	0.9998	0.9999	0.9999
LOD (μg/L) (S/N = 3)	5.0	20	20

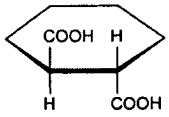
a) Consecutive runs (n = 6)
b) Consecutive days (n = 3)
c) Concentration range (0.05–1.0 mg/L)

3.4 Separation of *cis*- and *trans*-geometrical isomers

Cis- and *trans*-divalent anionic geometrical isomers could be also analyzed with the method presented here though optimization of the buffer pH was needed. The chemical structures and pK_a values of the analytes are shown in Fig. 4 and Table 3. The SIM electropherograms (Fig. 5) were obtained by monitoring the *m/z* 171 ion, which is corresponding to its deprotonated ion form, $[M-H]^-$. The separation of *cis*- and *trans*-cyclohexane-1,2-dicarboxylic isomers was investigated with acidic (pH 3.5, 4.0, 4.5) and alkaline running buffers (pH 9.0, 9.5, 10.0) (data not shown). It was found that the isomers comigrated when alkaline buffers were used. In contrast, good separations were observed with acidic buffers, particularly the pH 4.0 buffer gave the best separation and peak symmetry for each isomer. When the standard solution of the *cis*-isomer at a concentration of 1 mg/L was analyzed, the presence of a small amount of *trans*-isomer was confirmed. Therefore, the presented CE-MS method could be applied to this kind of impurity analysis with superior sensitivity and selectivity of MS. So far, a relatively alkaline volatile buffer has been used in CE-MS analysis of small anions to produce sufficient EOF towards the MS side for carrying all anions when a bare fused-silica capillary was used [21, 22]. However, as shown in Fig. 5, the



***cis*-1,2-cyclohexane dicarboxylic acid**



***trans*-1,2-cyclohexane dicarboxylic acid**

Figure 4. Molecular structures of the analytes.

Table 3. pK_a values of cyclohexane dicarboxylic acid isomers[a]

Substance	pK_1	pK_2
cis-Cyclohexane-1,2-dicarboxylic acid	4.23	6.77
trans-Cyclohexane-1,2-dicarboxylic acid	4.18	5.93

a) pK_a values were taken from *Lange's Handbook of Chemistry*, 11th ed., McGraw-Hill, New York 1973.

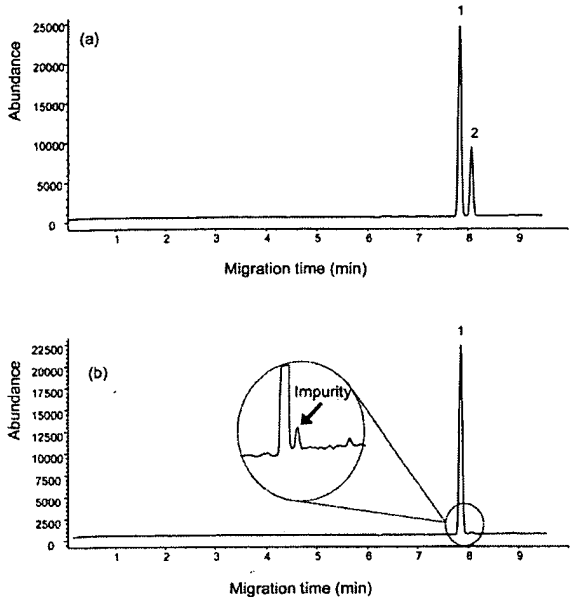


Figure 5. Separation of cyclohexane-1,2-dicarboxylic *cis-trans* isomers. (a) Isomer mixture, 1 mg/L each, (b) *cis*-isomer, 1 mg/L; running buffer, 20 mM ammonium acetate, pH 4.0; sample concentration, 1 mg/L each; peak identification: (1) *cis*-cyclohexane-1,2-dicarboxylic acid; (2) *trans*-cyclohexane-1,2-dicarboxylic acid. Other analytical conditions as in Fig. 1.

obtained result demonstrated that an acidic volatile buffer solution could also be used for anion analysis and the use of an alkaline sheath liquid gave enough ESI sensitivity even when an acidic running buffer was used. This result would extend the applicability of CE-MS analysis using bare fused-silica capillaries.

4 Concluding remarks

We have developed a simple, robust, and cost-effective universal CE-MS method for the analysis for small anionic isomers like anionic positional and geometrical isomers using a common uncoated fused-silica capillary and a simple composition of alkaline volatile running buffer. It was found that the buffer pH and sheath liquid pH have significant effects on the separation selectivity in CE and the sensitivity enhancement in ESI-MS. Positional isomers of hydroxybenzoate could be analyzed with alkaline buffer solution. *Cis-trans* geometrical isomers of cyclohexane dicarboxylic acid could be also separated and detected by the CE-MS method with acidic buffer solution though the EOF was relatively slow compared with alkaline buffer solution. Therefore, our results clearly indicated that the developed method could be applied to other small anionic isomers and also to other small anionic compounds regardless of isomers, which are difficult to analyze by common LC-MS methods.

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